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Susceptibility of some stone and pome fruit rootstocks to crown gall

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Summary. The susceptibility of different fruit rootstocks to crown gall disease was investigated in greenhouse and field experiments with numerous strains of *Agrobacterium tumefaciens* over three years. Plants were inoculated in the roots and shoots for pot experiments. Field experiments were performed in a naturally contaminated nursery plot. The genotypes *Prunus dulcis* and *P. persica* showed a high level of susceptibility to *A. tumefaciens*. Among the stone rootstocks, bitter almond was highly susceptible in all experiments. Apricot and Cadaman rootstocks displayed low susceptibility but larger galls, showing that there was no relation between rootstock susceptibility and gall size. Among pome rootstocks, quince BA29 was resistant to the disease, while MM106 was susceptible in potted trials; however, in the field essays, pome rootstocks did not become galled, possibly because the strains had selected for and adapted to stone rootstocks.

Key words: *Agrobacterium tumefaciens*, stone rootstock, pome rootstock, susceptibility.

Introduction

Agrobacterium spp. are ubiquitous telluric bacteria that infect dicotyledonous plants from some 100 plant families (DeCleen and De Ley, 1976), including economically important fruit and nut crops, grapes, and ornamental and landscape plants.

Crown gall is caused when the *Agrobacterium* species infecting the plant contains a large tumor inducing (Ti) plasmid (Ream, 1989). Gall formation results from the integration of a segment of Ti plasmid (T-DNA), into the plant cell genome (Gelvin, 1992). Inside the plant cell, genes in the T-DNA are expressed and lead to the synthesis of hormones (auxin and cytokinines) and to unusual compounds termed opines. Opines play a major role in the epidemiology of crown gall and the ecology of *Agrobacterium* spp. They serve as carbon and nitrogen sources for the tumor-inducing bacterium and some strains induce conjugal transfer of the Ti plasmid to the neighbouring non-tumorigenic agrobacteria (Dessaux *et al*., 1992).

Crown gall is a chronic and resurgent disease that causes significant economic losses in nurseries and orchards. The disease causes severe annual losses to growers and nursery men worldwide in

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the form of unsaleable nursery stock, low productivity from galled trees, and increased susceptibility of infected plants to other pathogens and to environmental stress generally (Bliss *et al*., 1999). Root pruning of rootstocks prior to transplanting to nursery fields is a routine practice, but it results in wounds that facilitate infection with tumorigenic agrobacteria that commonly inhabit nursery soils. Crown gall stunts mature plants by reducing root size and/or disrupting the vascular flow in the stems (Moore *et al*., 2001).

Although the epidemiology of crown gall has been the subject of extensive research in this and the last century, prevention strategies have, for the most part, remained focused on prophylactic methods such as carefully following cultural practices, strict inspection of nursery stock, using sterilised tools when grafting, and not planting in infected soils. The use of the antagonistic bacterium *Agrobacterium radiobacter* K84 as a preplanting preventive treatment has been successful for many years, and more recently a genetically engineered derivative of K84 named K1026 has become commercially available (Cooksey and Moore, 1982; Lopez *et al*., 1987; Jones and Kerr, 1989; Farrand, 1990; Penalver and López, 1999; Rhouma *et al.,* 2004). However, these antagonists are only efficient against susceptible strains of *Agrobacterium tumefaciens*. In spite of the success of traditional control strategies, crown gall still causes serious damage, notably in nurseries, in which the percentage of infected plants reaches more than 50% in some cases.

An alternative method of crown gall control is with the use of resistant rootstocks. Rootstocks with low sensitivity to the bacterium can benefit nurseries by reducing plant cullage and the cost of rootstock production. Resistant rootstock genotypes are of interest in breeding programmes. Genotypes resistant to *A. tumefaciens* have been described in different plant hosts such as aspen (Beneddra *et al*., 1996), chrysanthemum (Miller *et al*. 1975), grape (Sule *et al*., 1994), and peach (Pierronet and Eyquard, 1993; Zoina and Raio, 1999).

In this work, we report on a three-year study to evaluate the susceptibility to *A. tumefaciens* of some stone and pome fruit rootstocks commonly cultivated in Mediterranean countries including Tunisia. Experiments were carried out in pots, and in the field in naturally contaminated soil.

Materials and methods

Plant material and bacterial strains

Rootstocks of diverse origin (Table 1) and with different physiological and agronomic characteristics were tested for their susceptibility to two *A. tumefaciens* reference strains, B6 and C58, and to some virulent strains of the same bacterium used in mixture or separately (Table 2).

Pathogenicity determination by PCR

DNA extraction

Extraction of genomic DNA was performed on 10 ml of overnight bacterial cultures of *Agrobacterium* grown in Luria Bertani (LB) medium. Total DNA was extracted with a Dneasy Tissue Kit (Quiagen, Courtaboeuf, France).

PCR reaction and primers

Primers F749 (5'-GCTAGCTTGGAAGATCGCAC-3') and F14 (5' GAACGTGTTTCAACGGT-

Table 1. Rootstocks used in the study.

Rootstock	Genetic origin
Bitter almond	*Prunus dulcis*
Fasciuneddu	*Prunus dulcis*
GF 677	*Prunus persica* × *P. dulcis*
GF 557	*Prunus persica* × *P. dulcis*
Marianna (GF 8.1)	*Prunus munsoniana* × *P. cerasifera*
Cadaman	*Prunus persica* × *P. davidiana*
St Lucie	*Prunus cerasus*
Cab 6P	*Prunus cerasus*
Apricot (Mech Mech)	*Prunus armeniaca*
Myrobolan	*Prunus cerasifera*
BA 29	*Cydonia oblonga*
MM106	M1 × "Northern Spy"

Table 2. Characteristics of the *Agrobacterium tumefaciens* strains used in the study.

Strain	Origin	Host
C58	USA	Cherry
B6	USA	Apple
AA8	Tunisia	Bitter almond
GF2	Tunisia	GF 677
Myr3	Tunisia	Myrobolan
P125	Tunisia	Pear
M8	Tunisia	Apricot
MS	Tunisia	Mixture strains isolated from different rootstocks

TCA-3 ') were used in order to detect the localised region between the genes *vir*B11 and *vir*G15' on the T-DNA (Nesme *et al*., 1989).

The PCR was performed with a 25 μl reaction mixture containing: 2.5 μl of DNA extract, 2.5 μl of buffer Taq (10×), 2.5 μl of DNTP'S, 0.75 μl of $MgCl_2$, 11 μl of H_2O ultra pure sterile, 0.5 μl of W%, 2.5 μl of each primer and 0.25 μl of Taq polymerase. Amplification was achieved in a Perkin-Elmer thermocycler according to the following program: an initial denaturation at 94°C for 7 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 15s, elongation at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR Products (5 μl per sample) were mixed with 5 μl of a deposit buffer (sucrose, bromophenol blue and TBE 5×) and set down on 1% agarose containing 1 μg ml^{-1} ethidium bromide. Migration was in a tampon TBE 1× by horizontal electrophoresis at 80 V for 90 min. The gel was photographed under UV (302 nm).

Opine detection

Opines extraction were extracted with the method of Dessaux *et al*. (1998). Tomato galls ranging in weight from 10 to 300 mg were placed in a microtube. Distilled water (3 mg g^{-1} of sample) was added, and the tubes were heated to 100°C for 10 min. Healthy tomato stem tissues was taken as the negative control. Soft tissue was crushed, briefly vortexed, and separated from the liquid phase by centrifugation. The supernatant was collected and rotary-evaporated at 40°C under vacuum. Plant extracts were spotted on high-quality chromatography paper (Whatman 3MM, Whatman International Ltd., Maidstone, UK), and subjected to high-voltage paper electrophoresis, at 3500 V. Detection was carried out according to the nature of the opines investigated.

Inoculum preparation

Agrobacterium inoculum for the root and shoot inoculations was prepared by suspending 24-h-old cultures grown on mannitol glutamate medium (Moore *et al*., 2001) in sterile distilled water to a final concentration of 10^8 cfu ml^{-1} (DO=0.4).

Rootstock inoculation

Root and shoot inoculations were carried out following the method of Zoina and Raio (1999).

Root inoculation

Root inoculations were carried out by immersing the roots in the bacterial suspension (10^8 cfu ml^{-1}) for 5 min. Inoculated rootstocks were compared with control plants dipped in sterile distilled water.

Shoot inoculation

Shoot inoculations were carried out at the internodes by placing 10 μl of the bacterial (10^8 cfu ml^{-1}) suspension on 1-cm-long longitudinal wounds made with a sterile scalpel. Wounds were covered with sterile cotton impregnated in water and aluminium to prevent drying. Control inoculations were made with sterile distilled water. Gall development was inspected after two months.

Pot experiments

2001 experiments

Rootstocks were root-inoculated as described above and transplanted to pots containing an *Agrobacterium*-free soil and then placed under greenhouse conditions. The two reference strains (B6 and C58) and a mix of local isolates were used for inoculation. Forty plants per rootstock and per *Agrobacterium* strain were considered. After eight months, the percentage of galled plants, and the gall diameters and weights were determined. The following rootstocks were tested: bitter almond, GF 677, GF 557, BA29, MM106, apricot and Cadaman.

2002 experiments

Shoots were inoculated separately with the local strains and with the reference strains C58 and B6. The following rootstocks were tested: bitter almond, GF 677, GF 557, MM106, BA 29, myrobolan, St. Lucie, apricot and Cadaman. Two months after inoculation, rootstock susceptibility was evaluated on the basis of the percentage of inoculated sites that had formed galls, and gall size.

Field experiments (2001–2003)

Field experiments were carried out in a field with severe natural contamination of *A. tumefaciens* in a nursery of the region of Chbika (Kairouan, Central Tunisia). Roots were pruned before transplantation to the plots in a randomised block scheme with four replications. Forty plants per rootstock per block were tested. The following rootstocks were used: bitter almond, Fasciuneddu, GF

677, GF 557, GF 8.1, Cadaman, myrobolan, Cap 6P, St. Lucie, apricot, BA29 and MM106. Nine months after plantation, the percentage of galled plants and the weight and size of the galls were determined.

Data analysis

Data were subjected to analysis of variance (ANOVA). The significance of the mean differences was determined by Duncan's test and responses were judged significant at the 5% level (P=0.05).

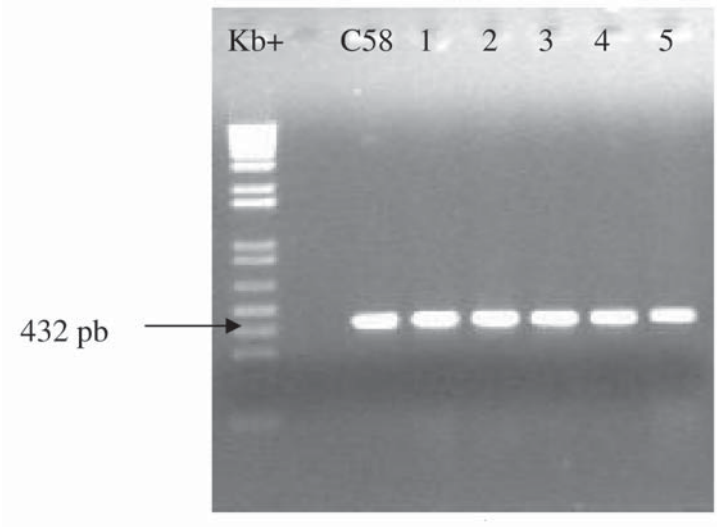

Fig. 1. Agarose gel electrophoresis (0.8%, 80 v) of the virulence region on pTi (*vir*B11-*vir*G15). Lanes 1–5, DNA from selected local isolates: 1, AA8; 2, GF2; 3, Myr3; 4, P125; 5, M8.

Results

Pathogenicity determination

All the strains tested induced galls on tomato 3 weeks after stem inoculation. PCR amplification of the region located between *vir*B11 and *vir*G 15 revealed a band with 432 bp for the isolates used in the study (Fig. 1).

Detection of opines

All the isolates tested were of the nopaline type (Fig. 2).

Pot experiments

The percentages of galled plants after root inoculation revealed that bitter almond, GF 677, GF 557 and MM106 were highly sensitive to crown gall (Fig. 3). Bitter almond rootstock was most susceptible, showing more than 90% of galled plants with C58, B6 and the mixture of local strains. Apricot rootstock appeared less susceptible to crown gall. Quince BA 29 and Cadaman rootstocks only rarely developed galls.

The mixture of local strains was more virulent than were the reference strains C58 and B6. Table 3 shows the gall diameters and weights recorded after inoculation with the reference strains and with the mixture of local strains. Voluminous galls were found on bitter almond, GF 677, GF 557, MM106 and Cadaman rootstocks. However, although Cadaman and apricot rootstocks showed only a low percentage of galled plants, the few galls that were recovered were voluminous and were similar to those obtained with the sensitive root-

Table 3. Gall size and weight after root inoculation (2001 experiment) (average data).

Rootstock	C58		B6		Mixed strains	
	Gall size (mm)	Gall weight (g)	Gall size (mm)	Gall weight (g)	Gall size (mm)	Gall weight (g)
Bitter almond	27.71 a	16.16 a	18.60 ab	5.93 a	29.77 a	19.79 a
MM 106	21.69 ab	13.19 ab	16.58 b	6.23 a	10.51 e	2.24 d
Cadaman	20.39 ab	12.18 ab			22.91 bc	9.98 b
GF 677	20.16 ab	6.97 bc	22.28 a	7.64 a	25.00 a	7.97 bc
GF 557	19.65 ab	7.47 bc	18.88 ab	6.22 a	23.06 bc	9.57 b
Apricot	15.27 bc	4.11 bc	17.51 b	4.85 a	17.47 cd	4.62 cd
BA 29	8.50 c	0.73 c			13.95 e	0.85 cd

Values in the same column followed by the same letter were not significantly different according to Duncan's multiple range test at P=0.05.

stocks. On BA 29 rootstock the gall diameters and weights were very small and significantly different from the gall diameters and weights on the other rootstocks.

The mixture of local strains (MS) was more virulent than the reference strains C58 and B6 as regards the percentage of galled plants. No correlation was found between gall diameter and strain virulence.

Shoot inoculation (2002 experiment)

Shoot inoculation of the rootstocks with the various strains of *A. tumefaciens* resulted in galls after two months. Except for the apricot rootstock, BA29 and Cadaman, all the strains formed tumors in more than 50% of the inoculation wounds. Bitter almond, GF 677 and GF 557 were highly susceptible to shoot infection by *A. tumefaciens* and formed tumors at more than 70% of the inoculated

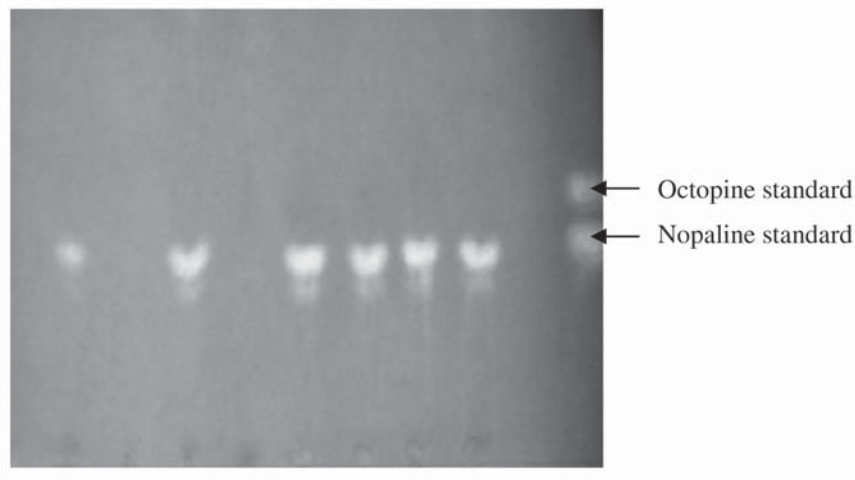

Fig. 2. Paper electrophoresis (3500 v) of opines from selected isolates.

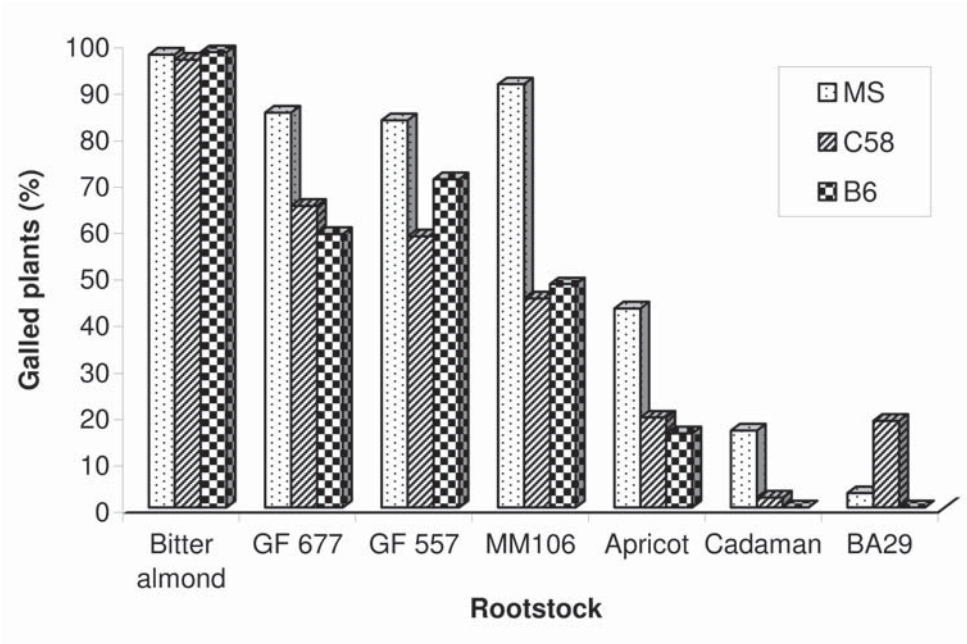

Fig. 3. Percentages of galled plants after root inoculation of rootstocks with *A. tumefaciens* strains MS, C58 and B6 (experiments in 2001)

sites (Fig. 4). Almost 50% of the inoculated sites were galled with Cadaman rootstock. However, with apricot and BA29 rootstocks, the percentage of galled sites did not exceed 25%. No particular strain-rootstock specialisation was observed but there were differences in virulence. Strain Myr3 isolated from myrobolan appeared the least virulent in terms of the percentage of galls it caused at the inoculation sites.

The mean gall size induced with the various virulent strains is shown in Table 4. All the strains induced galls on all the rootstocks, but on apricot and BA29 gall diameters were smaller and significantly different from those on other rootstocks. The largest galls occurred on the most sensitive rootstocks: bitter almond and almond × peach.

Field experiments

On all rootstocks tested, the percentage of galled plants increased every year during the three years of the experiment (Fig. 5).

Results clearly demonstrated the very high sus-

Table 4. Means of tumor size (mm) two months after shoot inoculations (2002 experiment).

Rootstock	B6	C58	Myr3	M8	AA8	GF5	P125
Bitter almond	13.50 a	15.20 a	4.30 bc	7.40 a	11.4 a	10.00 ab	17.50 a
GF 677	8.90 c	5.08 e	5.60 a	5.70 b	9.9 ab	11.40 a	13.90 c
GF 557	11.80 b	9.60 c	5.27 ab	5.72 b	9.9 ab	7.50 c	15.25 bc
Myrobolan	9.80 c	13.40 b	3.20 c	7.10 a	8.4 b	7.40 c	14.90 bc
MM106	10.08 c	11.60 c	3.98 bc	7.40 a	8.7 b	9.60 b	16.26 ab
St Lucie	5.00 d	8.50 d	4.36 bc	7.90 a	9.9 bc	6.90 c	16.30 ab
Cadaman	5.60 d	1.87 f	1.73 d	5.76 b	4.2 c	6.30 c	7.60 d
Apricot	2.50 e	1.38 f		7.50 a			
BA29	2.40 e	2.60 f	2.20 d				

Values in the same column followed by the same letter were not significantly different according to Duncan's multiple range test at P=0.05.

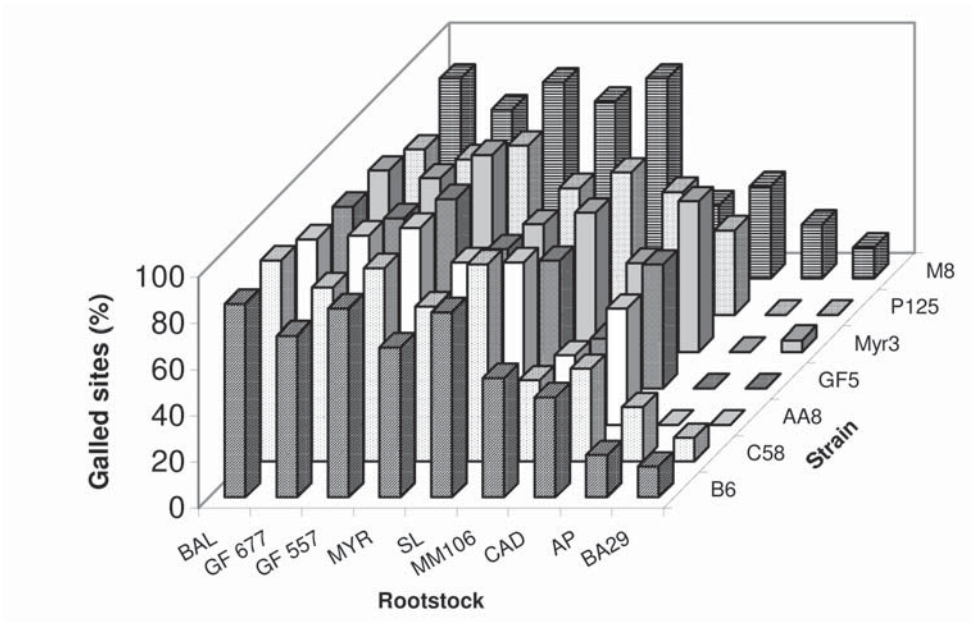

Fig. 4. Galled inoculation sites (%) after shoot inoculations with various strains of *Agrobacterium tumefaciens*. BAL, bitter almond; SL, Saint Lucie; MYR, myrobolan; CAD, Cadaman; AP, apricot.

ceptibility of the bitter almond rootstock, and the high susceptibility of the GF 677, GF 557, GF 8.1. and Fasciuneddu rootstocks. The percentages of galled plants with these rootstocks were more than 50% and reached 86% with the bitter almond rootstock. When uprooting the rootstocks, we noted that two of the rootstocks, GF 677 and GF 557 had suffered considerable damage from root-knot nematodes, facilitating the invasion of *Agrobacterium* into the roots. Apricot rootstock appeared more tolerant to crown gall than the other rootstocks. However, although MM106 rootstock was susceptible to crown gall in the pot experiments, this rootstock did not develop galls in the field experiments. No significant correlation was found between the rootstock susceptibility found with artificial inoculation and sensitivity in a naturally contaminated soil. The correlations between the percentage of galled plants in the pot tests and the field tests in 2001, 2002 and 2003 were 0.52, 0.51 and 0.56 respectively.

As shown in Table 5, gall diameters ranged from 19 to 25 mm. A comparison of the means with Duncan's test showed a maximum of three significantly different groups. Although the Cadaman and apricot rootstocks showed only a low percentage of galled plants, yet gall size with those rootstocks was similar to that of the more susceptible rootstocks.

Discussion

The resistance of rootstock genotypes to crown gall deserves study as it may help reduce plant cullage and costs of plant production due to the disease, and it is also of interest in breeding programmes. Sources of resistance have been report-

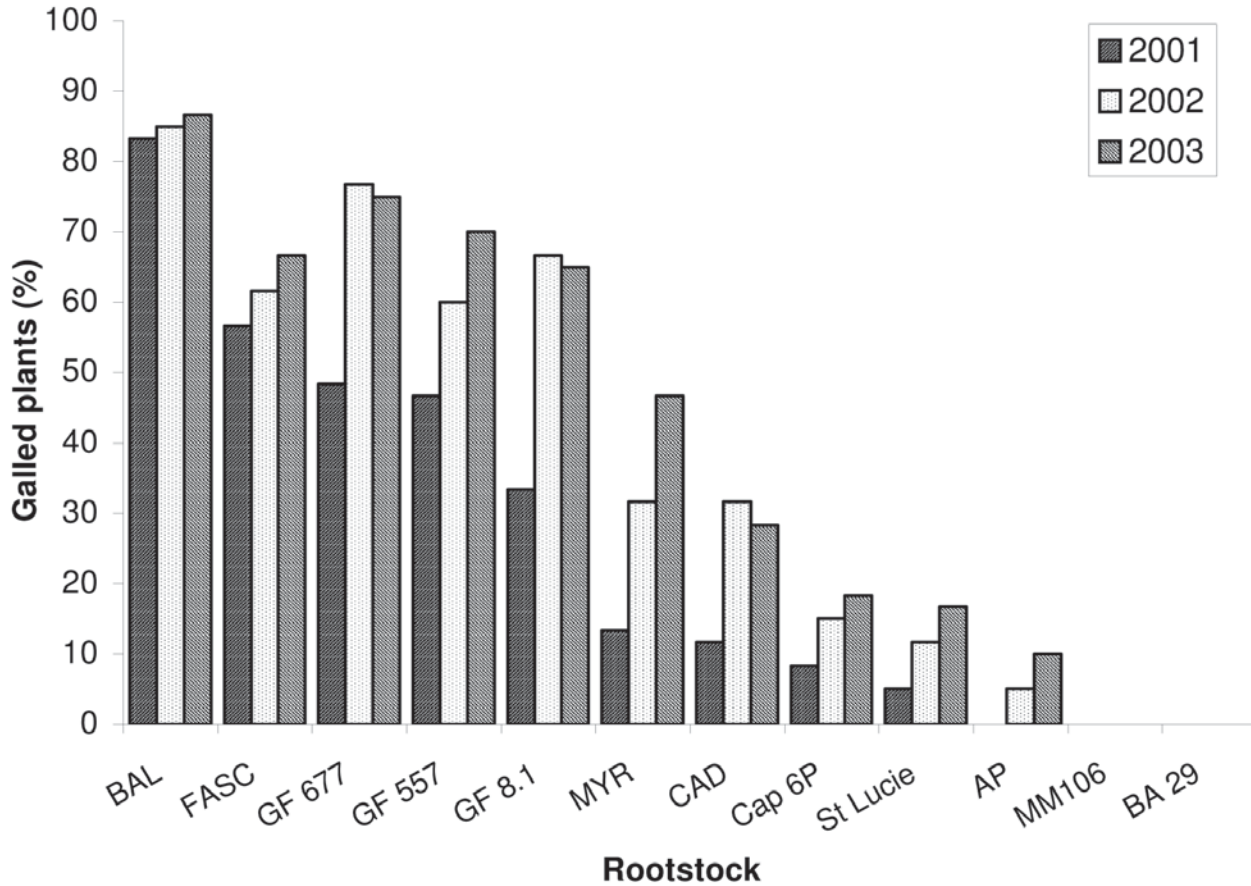

Fig. 5. Percentage of galled plants after growing in a naturally contaminated soil in 2001, 2002 and 2003. Legend: BAL, bitter almond; Fasc, Fasciuneddu; MYR, myrobolan; CAD, Cadaman; AP, apricot.

Table 5. Mean gall diameter and weight after growing in *Agrobacterium tumefaciens* contaminated soil.

Rootstock	Mean gall diameter (mm)			Mean gall weight (g)		
	2001	2002	2003	2001	2002	2003
Bitter almond	19.08 ab	18.11 ab	18.26 b	4.56 b	3.89 ab	5.30 ab
Fasciuneddu	17.07 bc	17.26 abc	19.36 b	4.53 b	3.32 ab	6.29 ab
GF 677	17.34 bc	17.55 abc	19.01 b	3.49 b	4.00 ab	4.07 bc
GF 557	15.23 bc	16.30 bc	18.77 b	2.49 b	3.55 ab	3.99 bc
GF 8.1.	19.29 a	17.85 ab	17.72 b	3.34 b	3.47 ab	2.83 c
Myrobolan	21.93 a	20.88 ab	25.95 a	2.52 b	5.00 ab	7.59 ab
Cadaman	21.90 a	21.33 ab	20.63 b	8.08 a	6.93 a	6.08 ab
Cap 6P	15.38 bc	15.80 bc	16.63 bc	2.36 b	3.30 ab	3.81 bc
St Lucie	13.66 c	15.38 c	16.03 bc	2.95 b	4.54 b	4.13 bc
Apricot		23.68 a	21.80 bc		5.53 ab	5.29 a

Values in the same column followed by the same letter were not significantly different according.
to Duncan's multiple range test at P=0.05.

ed in different plant species, but very little is known about the susceptibility of stone and pome rootstocks to *A. tumefaciens*. In the present study the susceptibility of some stone and pome fruit rootstocks commonly grown in Tunisia to various *A. tumefaciens* strains was evaluated.

Using PCR to detect the localised region between *vir*B11 and *vir*B15 on T-DNA was very useful to determine pathogenicity indirectly since the strain that induced galls on indicator plants produced the expected 432 bp band. The primers F14*vir*G15 and F749*vir*B11 should be used instead of the specific primers of nopaline and octopine.

Experiments both in the greenhouse and in the field showed that almond and peach rootstocks (bitter almond, Fasciuneddu, GF 677, GF 557) were highly susceptible to *Agrobacterium* strains and consequently to crown gall. The bitter almond rootstock appeared the most sensitive. This susceptibility was related to the two genotypes *P. dulcis* and *P. persica*. Similar results are reported by many authors (Zoina and Simeone, 1989; Pierronnet and Salesses, 1996; Zoina and Raio, 1999). Cadaman was more resistant than bitter almond and than the hybrids of peach and almonds. Because *P. persica* is a susceptible genotype, the relative resistance of Cadaman rootstock should be due to its *P. davidiana* genes.

The prune rootstock myrobolan *(P. cerasifera)* was susceptible to crown gall in pots and in the field experiments. Galls formed in the roots but never in the crown. Pierronnet and Salesses (1996) likewise reported that *P. cerasifera* and all the hybrids issued from crosses of which *P. cerasifera* was a member were highly susceptible to crown gall. These authors found that the genotype *P. domestica* presented low susceptibility to crown gall.

Field experiments in 2001, 2002 and 2003 were carried out in a nursery heavily contaminated with crown gall. The experimental plot had been used to grow almond and peach plants for many years. One surprising finding in these three years was that no pome rootstock became galled, not even MM106 which had been highly susceptible with root and stem inoculations in the pot trials. This could be explained as being caused by a process of soil selection for virulent bacteria adapted to almond and peach rootstocks. It was consistent with a previous study on biological control with K84 and K1026 (Rhouma *et al*., 2004). Repeated cultivation of pome rootstocks in the same plot probably allowed tumorigenic bacteria to adapt to these rootstocks so that more galls developed on them after three years. Deng and Nester (1998) reported that *Agrobacterium* spp. exhibited species and sometimes host specificity. Certain hosts may not have been sensitive to the phytohormone alterations brought about by the T-DNA, and therefore failed to form galls, while others respond-

ed with a necrotic reaction. It is also possible that extracts from roots of MM106 caused the resistance of this rootstock in the field. Belanger *et al*. (1995) reported that acetosyringone caused a mutation of tumorogenic strains that made them avirulent.

At the time of uprooting, a heavy infestation by nematodes on GF 677 and GF 577 rootstocks was observed; this facilitated invasion by *A. tumefaciens* and increased the percentage of galled plants. Karimi *et al*. (2000) also found that nematodes facilitated the entry of *Agrobacterium* into the plant roots. These authors inoculated two-week-old *Arabiodiopsis thaliana* with a mixture of *Meloidogyne incognita* and *A. tumefaciens* and found that during the nematode migration, the T-DNA was transferred into the root cells.

The quince BA 29 and apricot rootstocks appeared more tolerant to the disease with a low percentage of galled plants and galled inoculation sites. The low susceptibility of these rootstocks was difficult to explain, but a number of possible reasons can be suggested:

1. tolerant rootstocks permitted the nuclear transport of T-DNA, but resisted T-DNA integration into the nucleus. Many authors reported that resistance to crown gall was related to the lack of integration of T-DNA into the nucleus (Sule *et al*., 1994; Nam and Gelvin, 1998). T-DNA integration is crucial for gall formation. This process is the result of the expression of *iaaM*, *iaaH* and *ipt* oncogenes located on T-DNA (Escobar and Dandekar, 2003). These oncogenes share high nucleotide sequence conservation across *A. tumefaciens* strains (Escobar *et al*., 2001, 2002).

2. Early cell division at and around the wound site appears to be critical for transformation to occur. In the case of some resistant monocotyledon species, cells around the wound differentiated into lignified or sclerified cells without apparent division. Several authors have suggested that integration of the T-DNA into plant nuclear DNA may depend on cell cycle events, in particular DNA synthesis (Binns and Constantino, 1998).

3. The amount of acetosyringone secreted by a fruit rootstock could explain its resistance to crown gall. Recently, Tan and co-authors (Tan *et al*., 2004) reported that resistance of roses to crown gall was related to the amount of acetosyringone secreted. Resistant roses secreted less acetosyringone derivatives than susceptible roses. These researches concluded that the resistance mechanism of crown gall disease was related at least in part to the exudation of acetosyringone derivatives.

No relation was found between rootstock susceptibility to crown gall and gall size. As reported in the results, the few tumors recorded with apricot and Cadaman rootstocks had fewer galls, but galls that formed were larger and similar in size to those on the most susceptible rootstocks. Zhou *et al*. (2001) likewise studied the resistance to crown gall of progenies of roses between resistant 'Pekcougel' and susceptible 'Dukat' and found no correlation between disease incidence and gall size in some of these progenies.

The shoot inoculation data were correlated with the data on root inoculations. This was consistent with Zoina and Raio (1999); who stated that shoot inoculation was a good way to test for sensitivity to crown gall, especially when a great number of genotypes have to be tested. Pierronnet and Salesses (1996) also found that inoculating hardwood cuttings with *A. tumefaciens* was a good method to determine rootstock susceptibility.

The results clearly showed that the rootstocks most commonly used in Tunisia and in other Mediterranean countries were sensitive to crown gall.

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